Role of Tissue Factor in Metastasis: Functions of the Cytoplasmic and Extracellular Domains of the Molecule

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Summary

Tissue factor (TF) is a transmembrane glycoprotein that complexes with factor VIIa to initiate blood coagulation. It was reported in an earlier study that expression of high levels of TF in a human melanoma cell line promotes metastasis, and that the cytoplasmic domain of TF is required for this metastatic effect. To analyze the functions of the cytoplasmic and extracellular domains of TF in metastasis, two TF mutants were constructed; in one mutant alanine was substituted for each of the three serine residues in the cytoplasmic domain, preventing phosphorylation; in the other mutant alanine was substituted for four key residues in the extracellular domain, preventing binding of factor VIIa and consequently eliminating the initiation of blood coagulation by the TF-VIIa complex. Melanoma lines expressing high levels of either mutant form of TF were weakly metastatic in SCID mice, indicating that phosphorylation of the cytoplasmic domain and formation of a complex with VIIa by the extracellular domain are required for the full metastatic effect of TF. It was also found that increasing TF expression in human melanoma cells does not increase expression of vascular endothelial growth factor or promote growth and vascularization of tumors derived from the melanoma cells, suggesting that TF acts by a mechanism other than angiogenesis to promote metastasis.

Introduction

Tissue factor (TF) is a transmembrane glycoprotein containing a short cytoplasmic domain (CD), a single transmembrane domain (TD), and an extracellular domain (ED) (1). The ED forms a complex with factor VIIa that initiates blood coagulation; the CD is not required for the procoagulant activity of TF (2). In an earlier study we showed that increasing TF expression in a human melanoma line, by transfection of a cDNA encoding normal human TF, resulted in an increased metastatic potential of the cells as tested in a SCID mouse model of metastasis. The increase in metastatic potential was substantially reduced when the cells were transfected with a cDNA encoding a truncated TF molecule containing the ED and TD but lacking the CD, indicating that the CD is required to obtain the full metastatic effect of TF (3). Similar results were reported from another study in which Chinese hamster ovary (CHO-K1) cells were used for transfection instead of human melanoma cells (4).

The role of procoagulant activity in the metastatic function of T was also examined by introducing mutations in the ED that interfer with the initiation of blood coagulation. The first ED mutant testec which was partially defective in procoagulant activity, showed th same strong metastatic effect as normal TF in transfected huma melanoma cells (3), suggesting that procoagulant activity might not b involved. However, in a subsequent study using an ED mutant wit virtually no procoagulant activity, the metastatic function of TF i CHO-K1 cells was markedly reduced, indicating that procoagular activity of TF is involved in metastasis (4). The low level of procoagular activity in the ED mutant used for the earlier study could have bee sufficient to support metastasis.

The experiments reported here were designed to obtain furthe information about the roles of the CD and ED in metastasis. The CI contains three serine residues that can be phosphorylated (5, 6). To tes for a role of CD phosphorylation in the metastatic effect of TF, a TI mutant was constructed with alanine substitutions for the serine residue in the CD, which eliminated the possibility of phosphorylating the CD To test for a role of the TF-factor VIIa complex in the metastatic effec of TF, another TF mutant was constructed with alanine substitutions fo four residues in the ED involved in binding factor VIIa, which preven binding of factor VIIa and consequently eliminated the procoagulan activity. The results obtained with these two mutants suggest tha the full metastatic effect of TF requires both phosphorylation of the CI and binding of factor VIIa to the ED, in agreement with the results o another study (4).

Many tumor cells synthesize and secrete vascular endothelial growt factor (VEGF) which binds to receptors on vascular endothelial cells resulting in vascularization of the tumor (7). It was reported tha increasing TF expression in a murine fibrosarcoma cell line, by trans fection of normal murine TF cDNA, increased expression of VEGF (8) indicating that TF induces VEGF expression in these tumor cells However, another study showed that the growth and vascularizatio of tumors generated in SCID mice by stem cells derived from TF nul mutant (TF-/TF-) mice or from wild type (TF+/TF+) mice wer indistinguishable, indicating that TF does not induce vascularization i these tumors (9). In experiments reported here, we show that increasing TF expression in human melanoma cells does not increase VEGF expression or promote growth and vascularization of tumors derivec from the melanoma cells, indicating that TF acts in other ways tc promote metastasis.

Materials and Methods

Reagents. A rabbit polyclonal antibody prepared against the extracellular domain of human TF (residues 1–219) was provided by Dr. Yale Nemerson

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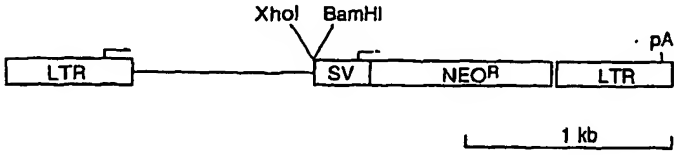

Fig. 1 Map of LXSN retroviral vector. LTR, long terminal repeat from Moloney murine leukemia virus; NEO^R, neomycin and G418 resistance gene; SV, SV40 promoter; pA, polyadenylation signal. The *Xho I* and *Bam* HI sites were used to ligate the cDNA inserts into LXSN. Arrows indicate transcriptional start sites and direction of transcription

(Mt. Sinai Medical Center, New York, NY). Hamster monoclonal antibody to mouse CD-31 was from Endogen Laboratories (Coburn, MA). Hamster IgG was from Pharmingen (San Diego, CA). Biotinylated goat anti-hamster IgG, normal goat serum and Vectastain ABC kit were from Vector Laboratories (Burlingame, CA). Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin solution, and trypsin-EDTA solution were from Sigma (St. Louis, MO). G418 was purchased from Gibco-BRL (Gaithersburg, MD). The cDNA encoding the 165 amino acid isoform of human VEGF ($VEGF_{165}$) was provided by Dr. Judith Abraham (Scios Nova Inc., Mountain View, CA).

Cell lines. All melanoma cell lines and the retroviral packaging cell lines PE501 and PA317 (provided by Dr. John Rose, Yale University School of Medicine) are described in an earlier report (3). The cells were grown in DMEM containing 10% FBS, penicillin, and streptomycin in a CO_2 incubator at 37° C. Cells were counted using a Coulter Counter ZM (Coulter Electronics, Hialeah, FL). Cultures were routinely monitored for mycoplasma contamination, which was not detected in any cultures.

Retroviral infection of melanoma cells. The retroviral vector LXSN (Fig. 1) was used to construct vectors for transfecting cDNAs encoding human TF, an ED mutant TF, two CD mutant TFs, and $VEGF_{165}$. The ED mutant TF has Ala substitutions at residues Lys 20, Asp 44, Asp 58 and Phe 140 to markedly reduce the affinity for binding of factor VIIa (10). One CD mutant TF is truncated after His 243 deleting the CD; the other CD mutant TF has Ala substitutions at residues Ser 253, Ser 258 and Ser 263 to eliminate phosphorylation of the CD. Titers of the retrovirus ranged from 10^3 to 10^6 colony-forming units per ml. The parental cell line YU-SIT1 (SIT1) was plated at a density of 5.0×10^5 cells in a 6 cm dish and grown overnight. On the following day the media in each dish was replaced with a retroviral stock (4 ml) supplemented with 16 µg Polybrene.

Table 1 Transfected human melanoma cell lines from parental line SIT1

Cell Line	Clonality	Proteins encoded by transfected cDNA insert
LSXN-1	C	None
LSXN-2	C	
LSXN-3	P	
LSXN-4	P	
TF-2	C	Normal Human TF
TF-4	C	
TF-5	P	
CD-1	P	Human TF with deletion after His243
CD-2	P	
4N-1	P	HumanTF with Ala substitutions at
4N-2	P	Lys20, Asp44, Asp58 & Phe140 in the ED
3SA-1	P	Human TF with Ala substitutions at
3SA-2	P	Ser53, Ser258 & Ser263 in the CD
VEGF-2	P	Human $VEGF_{165}$
VEGF-3	P	

C=Clonal P=Polyclonal

The cells were incubated for 24 h, replated in culture media containing 1 mg/ml G418 and incubated until G418-resistant transfected colonies were formed. The colonies were isolated and cultured to generate the cloned lines *TF*, *4N* (ED mutant), *CD* (CD mutant), *3SA* (CD mutant), and VEGF (Table 1).

Cell surface TF expression. Cell surface expression of TF was analyzed by flow cytometry as previously described (3). The procedure involved growing the cells as an attached monolayer, suspending the cells with trypsin, washing with phosphate-buffered saline (PBS), and resuspending the cells for 20 min at 4° C in PBS containing polyclonal rabbit anti-TF or rabbit IgG as a control. After washing the cells with PBS to remove unbound antibody, the cells were incubated for 20 min at 4° C with goat anti-rabbit IgG conjugated to fluorescein (Sigma), washed with PBS and analyzed on a Becton Dickinson FACS Star Analyzer.

Procoagulant activity. Cells were briefly incubated with trypsin-EDTA solution (Sigma) for 1.5 min, suspended in DMEM/10% FBS, pelleted, resuspended in Tris-buffered saline/0.1% bovine serum albumin at a density of 10^6 cells per ml, and disrupted by three cycles of freeze-thawing. The TF activity was determined by using a two-stage clotting assay (11) using factor X deficient human plasma (Sigma). Clotting times were converted to functional TF molecules by using a standardized curve prepared as described (12).

VEGF expression. Cells were plated in multi-well culture plates at a density of 10^5 cells per well. After 20 hr of incubation in DMEM supplemented with 10% FBS, the media was harvested and the concentration of VEGF was determined using an ELISA kit (R&D Systems, Inc., Minneapolis, MN).

Murine model of metastasis. Female 6-week-old SCID CB-17 mice (Charles River Laboratories, Wilmington, MA) were kept in isolated housing and received trimethoprim-sulfamethoxazole in their drinking water for Pneumocystis carinii prophylaxis (13). Cultures of the transfected melanoma lines were briefly trypsinized, suspended in DMEM/10% FBS, washed and resuspended in HBSS (free of calcium and magnesium). Mice were injected intravenously via the lateral tail vein with 4.0×10^5 cells. After 10-11 weeks the animals were euthanized, the lungs removed and the lung surface examined with a dissecting microscope for the presence of tumor nodules (3).

Murine model for primary tumor growth. Transfected cell lines were harvested, washed and resuspended in HBSS as described (3), and 0.1 ml containing 10^6 cells was injected subcutaneously into the flank of 6 week old female SCID CB-17 mice. The animals were euthanized after 3 weeks, and the tumors resected and weighed. The tumors were embedded in O.C.T. compound (Sakura Finetek, Torrance, CA), then frozen sections were cut and incubated with a primary hamster anti-mouse CD-31 (PECAM-1) antibody followed by biotinylated goat anti-hamster antibody. Staining to determine vascularity was performed as described using the Vectastain ABC kit with diaminobenzidine/H_2O_2 as substrate.

Statistical analysis. Data from the SCID mouse model of metastasis assay were analyzed by the two-tailed Fisher exact test. Primary tumor growth data were analyzed by an unpaired t-test. $P < 0.05$ was considered significant.

Results

For the first set of experiments, cell lines were constructed by retroviral-mediated transfections of the human melanoma line SIT1, which expresses a relatively low level of TF and is weakly metastatic in a SCID mouse model of metastasis (3). The TF cDNA inserts in the retroviral vectors for four of the transfected cell lines are shown in Table 1; the retroviral vector for the LXSN line, which serves as a control, does not have an insert. In the CD line the transfected TF molecule was truncated after residue 243, deleting the entire CD. In the *3SA* line, the three serine residues in the CD of the transfected TF molecule were changed to alanine, preventing phosphorylation of the CD. In the *4N* line, four residues in the ED of the transfected TF molecule were mutated, preventing complex formation with factor VIIa. Expression of the transfected TF molecules in the *TF*, *CD*, *3SA* and *4N* lines was measured by fluorescence-activated flow cytometry, using an anti-TF antibody (Table 2). The fluorescence intensity in each line was 10 to 15

times greater than in the *LXSN* line, indicating that the transfected TF molecule is strongly expressed in each line. The procoagulant activity in the *TF, CD* and *3SA* lines, as measured by a two-stage clotting assay, was 300 to 500 times greater than in the *LXSN* line (Table 3), consistent with other evidence that the CD is not required for procoagulant activity (2). The procoagulant activity in the *4N* line was about the same as in the *LXSN* line, indicating that the mutations in the ED eliminate procoagulant activity.

The five transfected lines were tested for their metastatic potential in a SCID mouse model of metastasis (Table 4). The incidence of metastasis was high (75%) for the TF line and low (23–33%) for the *CD, 3SA,* and *4N* lines. The results with the CD and 3SA lines indicate that the cytoplasmic domain is required for the full metastatic function, and that at least one of the serine residues in the CD is required for the full metastatic function, presumably because phosphorylation of the CD is important. The results with the *4N* line indicate that formation of a TF-VIIa complex is also required for the full metastatic function.

The second set of experiments was designed to determine whether TF can induce expression of VEGF, as suggested by another study using a murine fibrosarcoma cell line (8). The level of expression of the 165 amino acid isoform of VEGF ($VEGF_{165}$) in the *TF* and *LXSN* lines, which differ in the level of TF expression (Table 2) but are otherwise isogenic, was compared with a control line transfected with a cDNA encoding $VEGF_{165}$. The level of $VEGF_{165}$ secretion in both TF and LXSN lines, was about 30-fold lower than in the VEGF line (Table 5), indicating that increasing TF expression does not result in an increase of $VEGF_{165}$ expression.

It was also reported that TF promotes vascularization of murine and human tumors (8, 14). To test for an effect of TF on vascularization of human melanoma tumors, primary tumors were generated by subcutaneous injection of the *TF* and *LXSN* lines into SCID mice, and the vascularity of the resulting tumors was determined by immunohistochemistry; the positive control was the isogenic melanoma line *VEGF*, which expresses a high level of $VEGF_{165}$ (Tables 1 and 5). The tumors derived from the *VEGF* line showed a marked increase in vascularity as compared to the tumors derived from the *LXSN* control line, in contrast to the tumors derived from *TF* lines which showed no increase in vascularity (Fig. 2). These results indicate that vascularization of a primary human melanoma tumor is promoted by $VEGF_{165}$ but not by TF, consis-

Table 3 TF procoagulant activity in transfected melanoma cell lines

Cell Line	Procoagulant Activity TF Molecules per Cell x 10^3
LXSN-3	1.02
LXSN-4	0.72
TF-5	301.
CD-1	390.
CD-2	502.
4N-1	1.32
4N-2	1.44
3SA-1	448.
3SA-2	502.

A two-stage clotting assay was used to measure clotting time in lysed cells (11). The clotting time was converted to TF activity, as previously described (3). Assays were done in triplicate for the cell lines and the results generally agreed to ± 10%.

Table 4 Metastases in SCID mice after intravenous injection of transfecte human melanoma cells

Cell Line	Number of Mice Injected	Number of Mice with Lung Surface Metastasis	Incidence of Metastasis	P Value*
LXSN-3	9	0		
LXSN-4	5	0		
	14	0	0%	–
TF-5	16	12	75%	<0.001
CD-1	5	2		
CD-2	4	1		
	9	3	33%	0.047
4N-1	6	1		
4N-2	7	2		
	13	3	23%	0.098
3SA-1	6	2		
3SA-2	5	1		
	11	3	27%	0.072

Female 5 to 6 week old SCID mice were injected intravenously via the lateral tai vein with 4.0×10^5 transfected SIT1 melanoma cells. After 10 to 11 weeks th animals were euthanized and the lungs were examined for the presence of tumo nodules on the lung surface. The number of lung nodules in mice that develope tumors on their lung surface ranged from 1 to 11. In mice that develope metastases, similar numbers of lung nodules per mouse were observed for the TF CD, 4N, and 3SA cell lines.

* P values determined using two-tailed Fisher exact test for normal TF or mutant TF cell lines compared to control (LXSN) cell lines.

Table 2 Flow cytometry analysis of transfected melanoma cell lines

Cell Line	TF Expression on Cell Surface[1]
LXSN-1	2.0
LXSN-2	1.9
LXSN-3	2.0
TF-2	25
TF-4	25
TF-5	23
CD-1	30
CD-2	30
4N-1	21
4N-2	21
3SA-1	29
3SA-2	30
VEGF-2	2.0
VEGF-3	2.0

1. Relative fluorescence intensity of cells stained with anti-TF antibody compared to IgG control antibody.

Table 5 $VEGF_{165}$ expression in transfected human melanoma cell lines

Cell Line[1]	$VEGF_{165}$ Secreted Into Culture Medium[2] (Mean +/- S.D.)
LXSN-1	232 ± 82
LXSN-2	245 ± 70
TF-2	166 ± 89
TF-4	264 ± 51
VEGF-2	7580± 2100
VEGF-3	7500 ± 1740

1. The parent cell line is SIT1.
2. Amount of VEGF (pg) in 1.0 ml of culture medium after incubation of 10^5 cells for 20 hours as determined by ELISA. Each value represents mean level from three separate experiments.

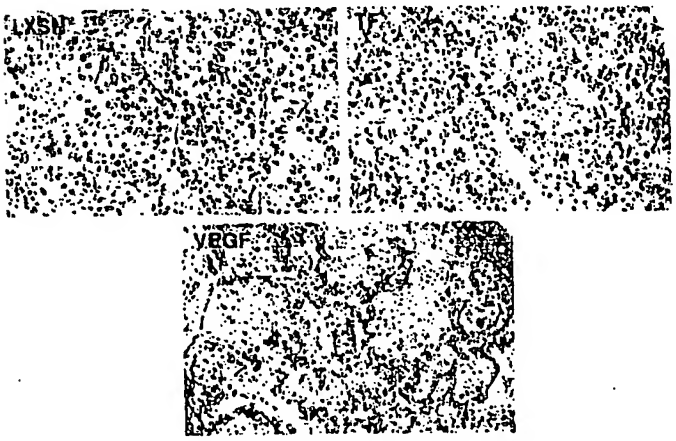

Fig. 2 Vasculature of primary tumors in SCID mice after subcutaneous injection of transfected human melanoma cells. Female 5 to 6 week old SCID mice were injected with 1.0×10^6 cells into the flank. Tumors were excised after 21 days and representative histologic sections shown after immunohistochemical staining for mouse CD-31. (×200)

tent with the finding that TF does not induce expression of $VEGF_{165}$. To test the effect of TF and $VEGF_{165}$ on the growth of primary tumors, transfected melanoma lines were injected subcutaneously into SCID mice and the weights of the tumors were compared 3 weeks after injection. The average weights of the tumors derived from the *TF* line and *LXSN* line were about the same, in contrast to a 5-fold average weight increase of the tumors derived from the *VEGF* line (Table 6).

Discussion

In an earlier study (3) it was shown that increasing TF expression by transfection of TF cDNA into a weakly metastatic human melanoma line resulted in a major increase of metastatic activity, and that the cytoplasmic domain (CD) of TF was required to achieve the full metastatic effect (3). In the study reported here it was shown that the role of the CD in metastasis depends on the presence of at least one of the three serine residues in the CD: When the melanoma cells were transfected with a TF cDNA encoding an alanine substitution for each serine, which eliminates the possibility for phosphorylation of the CD (5, 6), the metastatic effect of TF was reduced, suggesting that the metastatic effect of TF depends in part on phosphorylation of the CD. Similar results were reported in another study in which CHO-K1 cells were used instead of human melanoma cells to test the role of the serine residues in the CD on metastasis (4). Although the function of the CD in metastasis remains to be determined, it could involve an interaction with a 280 kD actin binding protein, which results in reorganization of actin filaments in the cytoskeleton (15).

We also tested for a role of the extracellular domain (ED) in the metastatic effect of TF, involving formation of a complex with factor VIIa that initiates the blood coagulation pathway. The complex with factor VIIa can be prevented by substitution of alanine for each of four critical residues in the ED (10). The metastatic potential of human melanoma cells expressing high levels of this ED mutant was reduced by greater than two-thirds, as compared to the cells expressing high levels of the normal TF, indicating that the complex of TF with factor VIIa increases the metastatic effect of TF. Similar results were reported in the other study in which CHO-K1 cells were used instead of human melanoma cells for the tests in SCID mice (4). It was further shown in that study that the metastatic effect of TF depends on the proteolytic activity of the TF-VIIa complex, suggesting that downstream components of the blood coagulation pathway are involved in mediating the metastatic effect of TF.

The next experiments were designed to test for a possible role of TF in the regulation of VEGF expression by tumor cells, as suggested by studies with a murine fibrosarcoma line (8). A panel of isogenic human melanoma lines expressing relatively low or high levels of TF and $VEGF_{165}$ was constructed, by transfection of TF cDNA or $VEGF_{165}$ cDNA into a parental line which is weakly metastatic in SCID mice. Increasing TF expression in the transfected melanoma cells did not increase $VEGF_{165}$ expression or the size and vascularity of the primary tumors derived from these cells. These results demonstrate that TF does not regulate $VEGF_{165}$ expression in cultured human melanoma cells, and does not promote angiogenesis in tumors derived from the cultured cells. This conclusion is supported by studies of transgenic mice carrying a TF transgene under control of an MMTV-LTR promoter, which generated high levels of ectopic expression of TF in salivary and mammary glands, but did not increase VEGF expression or vascularity in either gland (16). Other evidence that TF does not promote tumor growth and vascularity comes from experiments in which embryonic stem cells, derived from TF null mutant (TF-/TF-) mice were implanted subcutaneously into SCID mice to form primary tumors: The tumors formed by the TF null mutant cells (TF-/TF-) did not differ in growth or vascularity from tumors formed by the wild-type (TF+/TF+) cells (9).

The physiologic function of TF, which was initially thought to be limited to blood coagulation (1), is now known to include metastasis and possibly other cellular functions (17). The results reported here and in another study (4) have shown that both the procoagulant and metastatic functions of TF depend on the formation of a complex involving the ED and factor VIIa, and share a requirement for at least one of the downstream components in the blood coagulation pathway. However, the metastatic function but not the procoagulant function of TF also depends on the CD, apparently involving phosphorylation of least one of the serine residues in the CD. A possible role for the blood coagulation pathway in metastasis is to induce phosphorylation of the CD, which could involve thrombin, a component of the blood coagulation pathway, that binds to cellular receptors, resulting in activation of a signaling pathway (18, 19). The possible role of thrombin in regulating CD phosphorylation and the role of the CD in metastasis, remain as major problems for future investigation.

Table 6 Growth of primary tumors in SCID mice after subcutaneous injection of transfected melanoma cell lines

Cell Line[1]	Number of Tumors	Tumor Wt. (mg) at 3 Weeks Post-Injection (Mean +/- S.D.)	P Value*
LXSN-1	9	103 (+/- 50)	—
TF-2	8	135 (+/- 81)	0.340
VEGF-3	9	506 (+/- 237)*	<0.001

1. The parent cell line is SIT1.

* P values determined using an unpaired t-test for TF-2 or VEGF-3 cell lines compared to control (LXSN-1) cell line.

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